Vol. 67: 201–207, 2005 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published November 28

Pathogenicity of *Vibrio penaeicida* for white shrimp *Litopenaeus vannamei*: a cysteine protease-like exotoxin as a virulence factor

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ABSTRACT: The pathogenicity of *Vibrio penaeicida* Strains KH-1 and AM101, their culture-free supernatant (CFS), and their protein fraction obtained by 40% of ammonium sulfate precipitation (PFs40) were assessed in experimental challenges against juvenile *Litopenaeus vannamei*. Live *Vibrio* cells, CFS, and PFs40 from the AM101 strain produced a significantly higher mortality ($p < 0.05$) compared to the KH-1 strain. Toxicity and median lethal doses (LD_{50}) of Fast Protein Liquid Chromatography (FPLC) products were evaluated on *L. vannamei*. The first FPLC fraction sample (A) from PFs40 of the AM101 strain displayed LD_{50} values of 1.68 and 5.61 µg protein ind.$^{-1}$, respectively. The second FPLC process from Fraction A showed a peak (A1) also with toxic effects to shrimp. PFs40, Fraction A, and Peak A1 showed a 38.5 kDa molecular band (SDS-PAGE), with activity on a gelatin protease zymogram. The lethal effect of PFs40 and Fraction A was inhibited by Proteinase K, $CuCl_2$, E-64, and heat (60 and 100°C) treatments, but was not inhibited by EDTA-Na_2, aprotinin, and soy trypsin treatments. These results and the zymogram inhibition test suggest the presence of a cysteine protease-like proteinaceous exotoxin as a dominant protease, secreted by *V. penaeicida* Strain AM101.

KEY WORDS: *Vibrio penaeicida* · Cysteine protease · *Litopenaeus vannamei* · Shrimp · Exotoxin · Pathogenicity

INTRODUCTION

In Mexico, as in other countries, shrimp *Litopenaeus vannamei* and *L. stylirostris* culture has become an important economic activity. The culture systems create a beneficial environment to some specific bacterial communities that are part of the aquatic microbiota and which may be a serious problem to shrimp production (Jiravanichpaisal et al. 1994). *Vibrio* spp. show a wide distribution, and some species have been associated with shrimp as a source of serious infections under culture conditions (Lightner 1996, Alvarez et al. 1998, Costa et al. 1998, Saulnier et al. 2000a). Shrimp mortality caused by *Vibrio* has been reported from negligible to 100%, mainly at the larval stage of development (Hameed 1995, Prayitno & Latchford 1995, Harris & Owens 1999).

Vibrio spp. show great variation in terms of pathogenicity associated with host species, developmental stage, bacterial dose, bacteria species and particular strains, and exposure time and stress (Lightner 1996, Saulnier et al. 2000a, Aguirre-Guzmán et al. 2001). Different *Vibrio* extracellular products (ECP) have been identified and proposed as putative virulence factors in the species pathogenic to shrimp (Liu et al. 1996, 1997, Lee et al. 1997, 1999a,b, Chen et al. 1999,

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Harris & Owens 1999, Liu & Lee 1999, Montero & Austin 1999). A thermo-labile cytotoxic factor was detected in the ECP from *V. penaeicida*, which produces 100% mortality in juvenile *Litopenaeus stylirostris* (Goarant et al. 2000). Two toxic fractions isolated from *V. parahaemolyticus* ECP by gel filtration also produce similar mortality rates in *Penaeus monodon* (Sudheesh & Xu 2001).

Proteolytic enzymes, such as cysteine and serine proteases, metalloproteases, and hemolysins, have been isolated from *Vibrio harveyi*, *V. anguillarum*, and *V. alginolyticus* (Stensvag et al. 1993, Lee et al. 1997, Harris & Owens 1999). *V. harveyi* produces an extracellular 38 kDa protein with protease, phospholipase, and hemolytic activities for *Penaeus monodon* (Liu et al. 1997), while its biological properties can be effectively inhibited by iodoacetamide, iodoacetic acid, N-ethylmaleimide, p-chloromercuribensoate, and p-chloromercurybenzene-sulfonic acid and blocked by $CuCl_2$ and $HgCl_2$ (Liu et al. 1996, 1997, Liu & Lee 1999). *V. alginolyticus* produces a 33 kDa extracellular alkaline serine protease, which induces lethal effects on juvenile *Marsupenaeus japonicus* (Lee et al. 1997), and its proteolytic activity is inhibited by PMSF and $FeCl_2$, and is partially inhibited by $CaCl_2$, $CuCl_2$, $CoCl_2$, $MnCl_2$, and $ZnCl_2$ (Lee et al. 1997).

Furthermore, previous studies show that cell-free supernatants (CFS) and a protein fraction (PFs) of *Vibrio penaeicida* are pathogenic to *Litopenaeus stylirostris* (Aguirre-Guzmán et al. 2003).

The aim of the present study was to characterize a cystein protease-like exotoxin, from *Vibrio penaeicida* Strains AM101 and KH-1 as a putative virulence factor for the American white shrimp *Litopenaeus vannamei*.

MATERIALS AND METHODS

***Vibrio* strains and culture conditions.** *V. penaeicida* Strains KH-1 (Ishimaru et al. 1995) and AM101 (Costa et al. 1998) were stored at –80°C and pre-cultured on Zobell agar medium (4 g l^{-1} peptone, 1 g l^{-1} yeast extract, 15 g l^{-1} agar, 250 ml distilled water, 750 ml artificial seawater) at 27°C for 24 h. To induce the production of ECP, each bacterial strain was cultured in brain heart infusion broth (BHI, Difco) in artificial seawater (2.3% w/v NaCl, 20 mM KCl, 5 mM $MgSO_4$, 2 mM $CaCl_2$), under constant orbital shaking at 20°C for 48 h (Goarant et al. 2000).

PF isolation and purification. All purification procedures were carried out at 4°C. Bacterial cells of *Vibrio penaeicida* Strains KH-1 and AM101 were separated from the CFS by centrifugation (1500 × *g* for 10 min). CFS were concentrated (1:40, v/v) using a capillary flow dialysis cartridge (Baxter, Model 23.08), followed by filtration through a 0.2 µm pore-size filter. Ammonium sulfate was added to the CFS at 40% saturation (Scopes 1998), and the protein precipitate (PFs40) was collected by centrifugation (30 000 × *g* for 30 min), dissolved in sterile distilled water, and dialyzed against distilled water for 72 h, using semi-permeable dialysis bags with a molecular weight limit of 12 000 Da. The protein solutions were lyophilized and stored at –20°C until used. The sterility of all CFS and lyophilized preparations was verified by spreading 100 µl of each sample on Zobell agar plates, followed by incubation at 27°C for a minimum of 24 h.

Lyophilized PFs40 of *Vibrio penaeicida* Strain AM01 was reconstituted in sterile artificial seawater. A 500 µl aliquot was applied onto a Superose 12 column (Pharmacia-LKB) equilibrated with sterile artificial seawater and fractionated by FPLC at a flow rate of 0.2 ml min^{-1} (absorbance unit [AU] 0.05). Fractions of 1.0 ml were collected, and each 280 nm absorbing peak was collected, pooled, dialyzed, lyophilized, and stored using the same protocol described earlier until further application in shrimp bioassays.

Further molecular sieving chromatography was performed on active fractions in a Superose 12 column under the same chromatographic conditions described above.

Protein quantification. Total protein values were assessed at 595 nm with a protein dye reagent from Bio-Rad (Bio-Rad Laboratories) as described by the manufacturer. Bovine serum albumin was used to design a standard curve.

Electrophoresis and protease zymogram. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the general method of Laemmli (1970), with a 4% (w/v) acrylamide-bisacrylamide stacking gel and a 10% separating gel (Sambrook et al. 1989). Electrophoresis of denatured samples and molecular weight standards (Bio-Rad Laboratories) to calibrate the gel was performed in a Protean II xi vertical electrophoresis system (Bio-Rad) at 20 mA (constant current) at the beginning of the run and at 30 mA after introduction and migration of the bromophenol blue tracking dye. The gel was stained with a 0.2% $AgNO_3$ solution (200 µg $AgNO_3$, 75 µl formaldehyde 30%, and 100 ml distilled water) (Harlow & Lane 1988), and molecular weights were calculated.

The extracellular proteolytic enzymes from *Vibrio penaeicida* Strain AM101 were identified by zymogram analysis (Lantz & Ciborowski 1997). Briefly, protein samples in Laemmli (1970) buffer were loaded on a SDS-polyacrylamide gel on a 10% separating gel with gelatin (10 mg ml^{-1}) and on a 3% acylamide stacking gel, at 20 mA (constant current) initially and

at 30 mA after the tracking dye entered the separating gel. After electrophoresis, the polyacrylamide gel was washed first with 2.5% Triton X-100 in distilled water for 1 h, then with 2.5% Triton X-100 in 50 mM Tris buffer, pH 7.4 (2.5%, w/v), for another 1 h, and finally with 50 mM Tris-HCl buffer, pH 7.4, for 1 h. Protease bands were identified by incubating the polyacrylamide-gelatin gels in Tris buffer containing 1 mM dithiothreitol at 37°C for 2.5 h. Finally, the gel was stained overnight with Coomassie blue R-250. Zymogram analysis in the presence of various protease inhibitors was also done to determine the nature of the proteolytic activity.

Experimental shrimp. Different groups of juvenile (1 to 4.7 g weight) shrimp *Litopenaeus vannamei* from a local commercial shrimp farm were transported to the Center for Biological Research (CIBNOR), La Paz, Mexico, experimental facilities. The farm was previously confirmed to be pathogen free by the National Aquatic Animal Health Program (CIBNOR-PRONALSA, La Paz, Mexico). Shrimp were acclimated to laboratory conditions in 100 l tanks supplied with filtered seawater (3 µm pore-size filter), aeration, and no substrate bed (sand) for at least 1 wk. Seawater during the experiments was maintained at pH 7.8 to 8.2, 26 to 28°C, and 35‰ salinity. Juvenile shrimp were fed ad libitum with a commercial shrimp feed containing 35% crude protein.

Shrimp exposure to live *Vibrio* strains. All experimental challenges were conducted in a restricted area, since *V. penaeicida* has not been reported in Mexico. *V. penaeicida* Strains KH-1 and AM101 were cultured in Zobell's broth at 30°C under orbital shaking. After 24 h of incubation, bacterial suspensions were serially diluted 10-fold with sterile artificial seawater (Saulnier et al. 2000a). Shrimp (1 to 1.2 g weight) were exposed by immersion in 10 l of filtered and aerated seawater containing 10^4 colony forming units (CFU) ml^{-1} of each *Vibrio* strain. After a 2 h challenge, groups of 15 shrimp were transferred to plastic tanks containing 20 l of filtered and aerated seawater. Control shrimp were treated as above except that the bacterium was not added during exposure periods. Three replicate tanks were used for each treatment. Shrimp were monitored up to 120 h, and mortality rate (%) was expressed according to the formula: [1 – (final number of organisms/initial number of organisms)] × 100.

Shrimp injected with PF preparations from *Vibrio penaeicida*. Two groups of shrimp (3.5 to 4.7 and 2.5 to 3.0 g weight) were injected with either 50 µl of dialyzed CFS $ind.^{-1}$ (40 µg ml^{-1}) or 25 µl of PFs40 $ind.^{-1}$ (20 µg ml^{-1}) of *V. penaeicida* Strains KH-1 and AM101. Another group of shrimp (2.5 to 3.0 g weight) was also injected with 25 µl of FPLC (Fast Protein Liquid Chromatography) fractions $ind.^{-1}$ (10 µg ml^{-1}) of PFs40 from *V. penaeicida* Strain AM101. Each sample was injected intra-muscularly between the third and fourth abdominal segments. Three control groups were used (uninjected, and injected with 50 or 25 µl of sterile artificial seawater or dialyzed BHI medium $ind.^{-1}$). Each experimental group (CFS, PFs40, and FPLC fractions) was transferred to plastic tanks with 20 l of filtered (3 µm) and aerated seawater at a density of 10 shrimp per tank. Four replicate tanks were used for each experimental group and their controls, and mortality (%) was evaluated over 72 h.

The median lethal dose (LD_{50}) toxicity was calculated according to Liu et al. (1996), Lee et al. (1997), and Sudheesh & Xu (2001). A total of 15 shrimp per dose (2.0 to 3.0 g weight) was challenged by injection with 25 µl of PFs40 $ind.^{-1}$ from *Vibrio penaeicida* Strain AM101 at different doses (1, 2, 3, to 10 µg protein ml^{-1}). Similarly, shrimp were injected with the FPLC toxic peak from PFs40, at different doses (1.1, 1.2, 1.3, to 2.0 µg protein ml^{-1}).

Effects of neutralization of the PF on juvenile shrimp. The putative toxic virulence factors contained in the PFs40 and the FPLC fraction from *Vibrio penaeicida* Strain AM101 were subjected to different neutralizing treatments before being injected intra-muscularly into shrimp (25 µl $ind.^{-1}$) at 20 or 5 µg protein ml^{-1}, respectively. Neutralizing treatments included: (1) heat treatment of samples at 40, 60, and 100°C for 10 min; (2) enzymatic digestion with Proteinase K (100 µg ml^{-1} for 1 h at 37°C); and (3) mixing with 3 protease inhibitors: soybean trypsin inhibitor (200 µg ml^{-1} for 1 h at 37°C), EDTA-Na_2 $2H_2O$ (0.4 mg ml^{-1}), aprotinin (4 µg ml^{-1} for 1 h at 37°C), $CuCl_2$ (5 mM for 30 min at 25°C), and trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane or E-64 (0.5 mg ml^{-1} for 1 h at 37°C) (Lee et al. 1997, Liu et al. 1997, Montero & Austin 1999). Control shrimp groups were injected with one of the chemical inhibitors alone at the same concentrations. Quadruplicate shrimp treatments were transferred to plastic tanks with 20 l of filtered (3 µm) seawater.

Statistical analysis. Mortality and protein quantification data were compared by analysis of variance, multiple range test (Duncan), and correlation analysis using the Statistica software. LD_{50} values were calculated using the method of Reed & Muench (1938).

RESULTS

Pathogenicity of live *Vibrio* strains and toxicity of CFS and PFs40

Significant differences ($p < 0.05$) in pathogenicity were observed in shrimp exposed to *Vibrio penaeicida* Strains AM101 and KH-1 (Table 1). Shrimp mortality at

120 h post-exposure was 58, 27, and 11% for AM101, KH-1, and control treatments, respectively. Shrimp injected with CFS and the PFs40 fraction from *V. penaeicida* Strain AM101 showed a significantly higher mortality ($p < 0.05$) compared to *V. penaeicida* Strain KH-1, and to negative control groups at 72 h post-injection (Table 1). The LD_{50} value for the PFs40 from *V. penaeicida* Strain AM101 was calculated at 5.61 µg protein ind.$^{-1}$.

Characterization of the PF from *Vibrio penaeicida*

For FPLC analysis, PFs40 from *Vibrio penaeicida* Strain AM101 was selected because of the significantly higher mortality rate displayed by juvenile shrimp *Litopenaeus vannamei* when injected with this fraction. Four major fractions (A, B, C, and D) were obtained from this first FPLC process. Only Fraction A displayed a significant ($p < 0.05$) toxic effect on shrimp, with a mortality rate similar to that of PFs40 of *V. penaeicida* Strain AM101 at 42 and 66 h, respectively (Table 2). The LD_{50} value of Fraction A to juvenile shrimp *L. vannamei* was calculated at 1.68 µg protein ind.$^{-1}$.

Fraction A was re-chromatographed on a Superose 12 column by FPLC, and 5 peaks (A1 to A5) were obtained (Fig. 1). Peak A1 displayed a significant ($p < 0.05$) and similar mortality rate as that of Fraction A and PFs40 (<95%) when it was injected intramuscularly into juvenile shrimp (Table 2). Peaks A3 and A4 were the highest peaks, but like Peaks A2 and A5 and the control treatment, these did not produce significant shrimp mortality ($p > 0.05$). The PFs40, Fraction A and Peak A1 showed a similar protein profile banding pattern, with a primary banding of approximately 38.5 kDa (Fig. 1B).

Table 2. *Litopenaeus vannamei.* Mortality rate (±SD) of juvenile shrimp intra-muscularly injected with 25 µl ind.$^{-1}$ of FPLC (Fast Protein Liquid Chromatography) fractions (10 µg ml^{-1}) of *Vibrio penaeicida* Strain AM101. Control[1]: shrimp injected with sterile artificial seawater; Control[2]: shrimp not injected). Different superscripted letters indicate different homogeneous subsets as defined by a Duncan multiple-range procedure ($p < 0.05$)

FPLC 1 (42 h)		FPLC 2 (66 h)	
Fraction A	100 ± 0[a]	Peak A1	95 ± 6[a]
Fraction B	0 ± 0	Peak A2	0 ± 0
Fraction C	0 ± 0	Peak A3	15 ± 6[b]
Fraction D	0 ± 0	Peak A4	3 ± 5[b]
PFs40	100 ± 0[a]	Peak A5	3 ± 5[b]
Control[1]	0 ± 0	Peak A	100 ± 0[a]
Control[2]	0 ± 0	PFs40	98 ± 5[a]
		Control[1]	5 ± 6[b]
		Control[2]	3 ± 5[b]

Vibrio penaeicida protease activity and zymogram

The toxicity and proteolytic activity of PFs40 and Fraction A from *Vibrio penaeicida* Strain AM101 were significantly inhibited by heat shock (10 min at 60 and 100°C), $CuCl_2$, E-64, and Proteinase K treatment, as indicated by *in vivo* testing on juvenile shrimp and zymogram analysis (Table 3). Control shrimp groups (injected with one of the chemical products) showed mortality rates between 0% and 2.5%. Zymograms of PFs40, Fraction A, and Peak A1 of *V. penaeicida* Strain AM101 all exhibited similar proteic activity on gelatin (Table 4). Those samples displayed a main proteolytic band of approximately 38.5 kDa (data not shown).

Table 1. *Litopenaeus vannamei.* Mortality rate (±SD) of juvenile shrimp exposed to live *Vibrio* cells or intra-muscularly injected with CFS (culture-free supernatant, 40 µg protein ml^{-1}) and PFs40 (protein fraction, 20 µg protein ml^{-1}) of *Vibrio penaeicida* Strains KH-1 and AM101. Control[1]: shrimp not exposed; Control[2]: shrimp injected with brain heart infusion medium; Control[3]: shrimp injected with sterile artificial seawater; Control[4]: shrimp not injected. Shrimp were injected with 50 µl of CFS or 25 µl of PFs40. Different superscripted letters indicate different homogeneous subsets as defined by a Duncan multiple-range procedure ($p < 0.05$)

Exposed treatment (120 h)		Injected treatment (72 h)		
Strain	Live *Vibrio* cell	Strain	CFS	PFs40
AM101	57.8 ± 7.7[a]	AM10	77.5 ± 9.6[a]	97.5 ± 5.0[a]
KH-1	26.7 ± 6.7[b]	KH-1	15.0 ± 5.8	60.0 ± 8.2[b]
Control[1]	11.1 ± 3.8	Control[2]	5.0 ± 5.6	2.5 ± 5.0
		Control[3]	7.5 ± 5.0	0 ± 0
		Control[4]	0 ± 0	0 ± 0

DISCUSSION

Vibrio species are pathogenic agents for a wide range of invertebrate organisms (Lightner 1996), and there are great variations in virulence. Those variations have been associated with different processes that affect the pathogen as well as the host. For example, Hameed (1995), Prayitno & Latchford (1995), Costa et al. (1998), Harris & Owens (1999), and Saulnier et al. (2000b) have demonstrated that virulence of *Vibrio* species can be associated with the strain of *Vibrio*, the route of infection, and specific host factors, such as species, age, and physiological state.

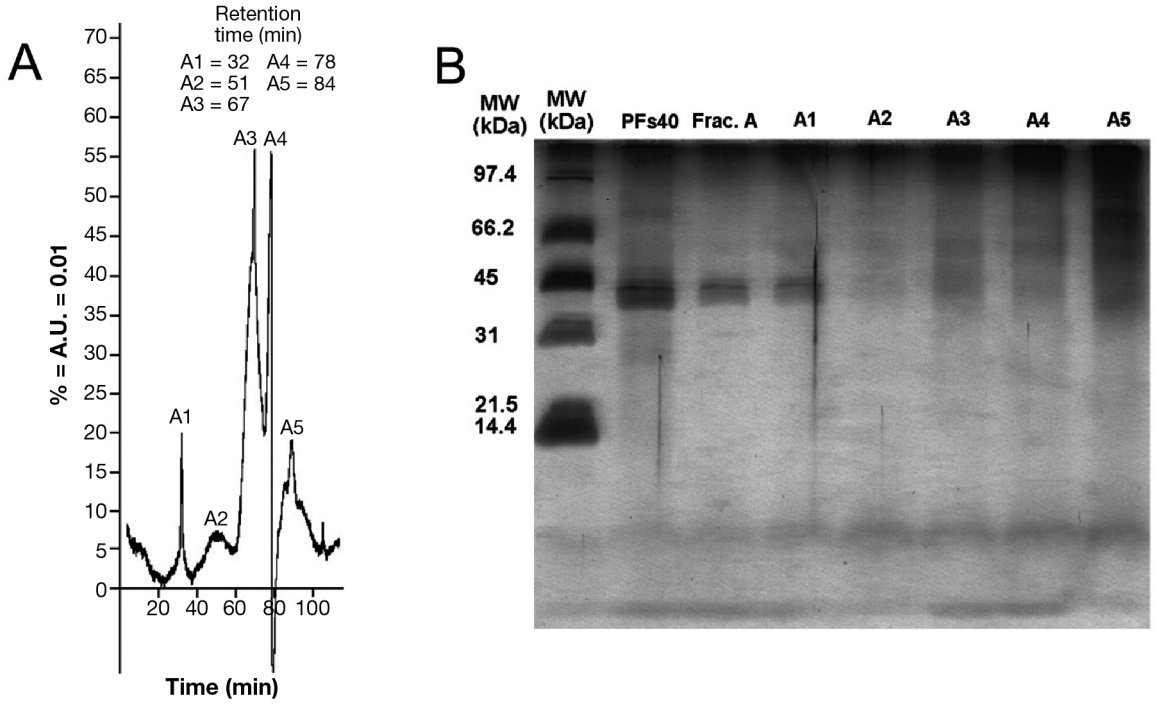

Fig. 1. (A) Chromatography peaks and (B) SDS-PAGE profiles of Fraction A from PFs40 of *Vibrio penaeicida* Strain AM101. SDS-PAGE (10%) after silver nitrate staining: molecular weight marker (MW, Lane 1), PFs40 crude (Lane 2), Fraction A (Lane 3) and FPLC peaks A1 to A5 from Fraction A (Lanes 4 to 8). A.U.: absorbance units

The present study shows that there are significant differences in the pathogenicity of *Vibrio penaeicida* strains (KH-1 and AM101) and the toxicity of their CFS and PFs40 products to *Litopenaeus vannamei*. Juvenile shrimp, 1 to 1.2 g weight, exposed to *V. penaeicida* Strain AM101 (10^4 CFU ml^{-1}) displayed a significantly higher mortality ($p < 0.05$) than shrimp exposed to Strain KH-1, after 120 h post-exposure. De la Peña et al. (1993) showed an LD_{50} of 10^2 to 10^3 CFU ml^{-1} (22 h post-exposure) for larger shrimp *Marsupenaeus japonicus*, 13 to 22 g weight, exposed to Strain KH-1, whereby Saulnier et al. (2000b) showed an LD_{50} of 10^4 CFU ml^{-1} (48 h post-exposure) for large (10 to 14 g weight), *L. stylirostris* shrimp, exposed to Strain AM101. This suggested a possible resistance of *L. vannamei* to these strains or a specific virulence of *V. penaeicida* against their original host as suggested by Saulnier et al. (2000a).

Table 3. *Litopenaeus vannamei.* Mortality rate (±SD) of juvenile shrimp intramuscularly injected with PFs40 and Fraction A of *Vibrio penaeicida* Strain AM101 after different inhibition treatments. Different superscripted letters indicate different homogeneous subsets as defined by a Duncan multiple-range procedure ($p < 0.05$). Prot. K: Proteinase K; EDTA: EDTA-Na_2 or ethylenediaminetetraacetic acid; Trypsin: soybean trypsin inhibitor; Not injec.: shrimp not injected; Seawater: shrimp injected with sterile artificial seawater; –: no mortality

	Inhibition treatment		Control group		
	PFs40	Fraction A		PFs40	Fraction A
$CuCl_2$	40 ± 8.2[a]	37.5 ± 5.0[a]	$CuCl_2$	2.5 ± 5.0	5.0 ± 5.8
E-64	37.5 ± 9.6[a]	37.5 ± 11.2[a]	E-64	–	2.5 ± 5.0
Aprotinin	92.5 ± 9.6[b]	97.5 ± 5.0[b]	Aprotinin	–	2.5 ± 5.0
Prot. K	2.5 ± 5[a]	5.0 ± 5.8[a]	Prot. K	–	–
EDTA	75 ± 12.9[b]	95 ± 5.8[b]	EDTA	2.5 ± 5.0	–
Trypsin	90 ± 8.2[b]	97.5 ± 5.0[b]	Trypsin	2.5 ± 5.0	–
40°C	95 ± 5.8[b]	92.5 ± 9.8[b]	Not injec.	–	–
60°C	17.5 ± 15[a]	10 ± 8.2[a]	Seawater	–	2.5 ± 5.0
100°C	7.5 ± 9.6[a]	2.5 ± 5.0[a]	PFs40	95 ± 5.8[b]	
			Frac. A		100.0 ± 0.0[b]

ECP from *Vibrio* sp. that have demonstrated toxicity to shrimp have been proposed as important elements of virulence. CFS and PFs40 of *V. penaeicida* Strain AM101 caused a significantly higher mortality ($p < 0.05$) in juvenile shrimp *Litopenaeus vannamei*. A similar protein profile in PFs40 of *V. penaeicida* Strains KH-1 and AM101 has been previously reported by Aguirre-Guzmán et al. (2003), whereby juvenile shrimp of *L. stylirostris* (2.5 to 4.0 g weight) injected with CFS and PFs40 from Strain AM101 displayed a higher mortality compared to Strain KH-1.

Table 4. Zymogram activity of PFs40 and Fraction A of *Vibrio penaeicida* Strain AM101. +++: high; ++: moderate; +: low; –: negative proteolytic activity on SDS-PAGE with gelatin (10 mg ml $^{-1}$; NE: proteolytic activity not evaluated; Prot. K: proteinase K, EDTA: EDTA-Na_2 or ethylenediaminetetraacetic acid; Trypsin: soybean trypsin inhibitor

Inhibition treatment	PFs40	Frac. A
$CuCl_2$	++	+
E-64	++	+
Aprotinin	+++	++
Prot. K	NE	NE
EDTA	+++	++
Trypsin	NE	NE
40°C	++	++
60°C	–	–
100°C	–	–

The LD_{50} values from Strain AM101 were comparable to those reported for CFS prepared from *V. parahaemolyticus* (8 µg ind.$^{-1}$) (Sudheesh & Xu 2001) and *V. harveyi* (4.4 µg ind.$^{-1}$) (Montero & Austin 1999). All LD_{50} values were obtained by intra-muscular injection of CFS in juvenile *Penaeus monodon*, *L. vannamei*, and *Nephrops norvegicus*. The Fraction A obtained from Strain AM101 produced a lower LD_{50} value (1.68 µg ind.$^{-1}$) than that reported by Harris & Owens (1999) for *P. monodon* after intra-muscular injection of T1 and T2 toxic fractions (1.8 and 2.2 µg g^{-1} body weight) obtained from the CFS of *V. harveyi*.

The Fraction A and PFs40 of *Vibrio penaeicida* Strain AM101 showed a similar heat- (60 and 100°C) and Proteinase K-induced inhibition of toxicity. This suggests that these factors are not endotoxins, such as lipopolysacharides, which are unaffected by such treatments (Montero & Austin 1999). These results agree with reports of a thermo-labile cytotoxic factor for juvenile shrimp *Litopenaeus stylirostris* from *V. penaeicida* Strain AM101 (Goarant et al. 2000), and 2 heat-sensitive protease fractions toxic for juvenile *Penaeus monodon* from the ECP of *V. parahaemolyticus* (Sudheesh & Xu 2001).

Chitinases, hemolysins, and proteases (cysteine protease, alkaline metal-chelator-sensitive proteases, serine protease, metalloprotease) have been isolated from CFSs of *Vibrio harveyi*, *V. anguillarum*, *V. alginolyticus*, etc. (Stensvag et al. 1993, Liu et al. 1996, 1997, Lee et al. 1997, Svitil et al. 1997, Harris & Owens 1999). Treatments, such as heat (40°C), EDTA-Na_2, and aprotinin on Fraction A and PFs40, had no effects on *in vivo* toxicity and zymogram analysis of these samples, suggesting that the metalloproteases or serine proteases are not the toxic factors responsible for the virulence of *V. penaeicida* Strain AM101. Only E-64 and $CuCl_2$ treatments, for both samples, showed a significant suppression effect ($p > 0.05$) of *in vivo* toxicity and zymogram analysis. These results suggest that a cysteine protease-like exotoxin is a virulence factor of this strain. Peak A1 of Fraction A, the Fraction A, and the PFs40 obtained from CFS of *V. penaeicida* Strain AM101 showed a similar protein band of approximately 38.5 kDa molecular weight. Liu et al. (1996, 1997) reported high toxicity of an ECP from *V. harveyi* isolated from diseased *Penaeus monodon* with protease, phospholipase, hemolysin activity. This activity was inhibited by iodoacetamide, iodoacetic acid, N-ethylmaleimide, p-chloromercuribensoate, p-chloromercurybenzene-sulfonic acid, $CuCl_2$, and $HgCL_2$. In addition, this ECP showed a purified cysteine protease (38 kDa), which could neutralize the clotting ability of normal prawn hemolymph (Lee et al. 1999a,b).

In summary, in agreement with previous reports, the present study shows the differences in virulence of live *Vibrio* cells and toxicity of CFS and PFs40 of *V. penaeicida* Strains AM101 and KH-1 to shrimp. It is conceivable that some products contained on PFs40 could be used as a virulence mechanism by *V. penaeicida*. Neutralizing treatment in *in vivo* tests on juvenile shrimp of *Litopenaeus vannamei* and zymogram analysis of PFs40 and Fraction A of Strain AM101 of *V. penaeicida* suggest the presence of a cysteine protease-like proteinaceous exotoxin as a dominant protease. The PFs40, Fraction A, and Peak A1 from Strain AM101 displayed a main proteolytic band of approximately 38.5 kDa. The presence of a cysteine protease-like proteinaceous exotoxin and the toxicity of Fraction A and Peak A1 from Strain AM101 deserve to be further investigated, to determine the relation between *V. penaeicida* infections and their effect on different shrimp species.

Acknowledgements. This study was funded by grants from the Mexican Council of Science and Technology (CONACyT Project 28884-B), the Cortez Sea Research System (SIMAC Project 980106033), the Center for Biological Research (CIBNOR, Project AC 1.7 to F.A.), and the International Foundation for Science (IFS, Project A/3229-1 to G.A.-G.). *Vibrio penaeicida*, Strain KH-1, was kindly provided by Dr. Leonardo Lizarraga (Centro de Investigación Científica y de Educación Superior de Ensenada). We are grateful to APSA in La Paz, Mexico, for supplying shrimp, and to Dr. J. Genaro Sanchez at Fac. de Medicina Veterinaria y Zootecnia, UAT, for English editorial advice.

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Editorial responsibility: Timothy Flegel, Bangkok, Thailand

Submitted: August 17, 2004; Accepted: June 2, 2005
Proofs received from author(s): November 4, 2005